2506-Pos Board B492
Adriamycin-Induced Mitochondrial Toxicity in Rat Heart is Exacerbate by Angiotensin
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Adriamycin (ADR) increases the production of reactive oxygen species, which diminishes mitochondrial function. Angiotensin-II stimulates mitochondrial-ROS generation. The aim of the study was to examine whether angiotensin converting enzyme (ACE) or renin inhibitors (captopril and/or aliskiren) protect against ADR-induced mitochondrial function impairment.
Rats were divided into five groups (n=14 each). The control group was treated with saline. ADR was administered to the four other groups every 2 days (4 mg/kg i.p). One of these was co-administered captopril (10 mg/kg/daily) and the other was co-treated with aliskiren (50 mg/kg/daily), while another was co-treated with both captopril and aliskiren (captopril and aliskiren were gavage administration daily for 8 days). Left ventricular function, ECG variables and blood pressure were assessed at the end of treatment period. The hearts were homogenized and biochemical measurements were made in mitochondria, cytosol and plasma. Mitochondria membrane potential (MMP), ATP levels were determined.
ADR decreased in the left ventricular developed pressure (LVDP), the maximal rate of rise of pressure (+dP/dt), and increased in the left ventricular end-diastolic pressure (LVEDP). ADR increased ST interval and decreased mean blood pressure. ADR increased oxidative stress in mitochondrial, cytosolic and plasma. ADR decreased MMP and ATP level in myocyte mitochondria. ADR co-administration with renin and ACE inhibitors improved the dissipation of MMP. The decreased in ATP level was restored by treatment with inhibitors of ACE and renin. By maintaining normal levels of mitochondrial MMP and ATP, captopril and aliskiren treatment prevented the pathologic changes in ECG, blood pressure and left ventricular function.
We concluded that inhibitors of angiotensin II are effective against ADR cardiotoxicity via the restoration of MMP and ATP production and prevention of mitochondrial damage in vivo.

Ion Motive ATPases

2507-Pos Board B493
Structural and Physiological Factors Controlling the Ion Specificity of ATP Synthase Membrane Rotors
José D. Faraldo-Gómez, Alexander Krah, Vanessa Leone, Florian Rössler.
ATP synthases are large multi-subunit complexes that utilize the energy stored in transmembrane electrochemical gradients of H(+) or Na(+) for the synthesis of ATP, through a rotary mechanism. A membrane-embedded sub-complex, known as the rotor ring, is the key structural component that transforms the free-energy gained from downhill ion transport into mechanical rotation. The rotor ring thus confers the ATP synthase with its ion specificity, influencing the degree to which cell-growth will be viable in a given physiological context. Here, we review our understanding of the principles that control the ion specificity of ATPase/synthase membrane rotors, based upon extensive theoretical work and biochemical and structural data for a range of representative organisms. In particular, we discuss how a conserved E/D side chain provides the ion-binding sites of all rotors across the F, V and A-ATPase/synthase subfamilies with a strong, intrinsic selectivity for protons. This default specificity, however, is somehow drastically enhanced or reduced to allow for actual H(+) or Na(+) coupling under physiological conditions. We show that such strong modulation is provided by the spectrum of non-conserved amino-acids that decorate the ion-binding sites. While hydrophobic side chains contribute to enhance the H(+) selectivity of the rotors to the extreme levels required in many cases, polar side-chains and structurally-bound water molecules have the opposite effect, and can make a binding site essentially non-specific - which facilitates Na(+)-coupling. Altogether, this analysis illustrates a process of adaptation in the chemical structure of an indispensable enzyme, so as to meet the requirements of a given physiological environment.

2508-Pos Board B494
Interactions Between the γ Subunit and the C-Terminal Domain of the ϵ Subunit Mediate ϵ Subunit Inhibition of F1-ATPase
Natalie C. Negrey, Robert K. Nakamoto.
The ϵ subunit of the F_1 sector of the *Escherichia coli* ATP synthase is part of the rotor complex (γ-ϵ-c_{10}). ϵ subunit plays important roles in energy coupling between H^+ transport and ATP hydrolysis/synthesis but also imparts an inhibitory activity on the catalytic mechanism. We characterize the structure/function effects of two γ subunit Cys substitutions that affect ϵ inhibition of ATPase activity. γT106C by itself has little affect, but labeling of the cysteine with methanethiosulfonate spin label (MTSL) blocks the ability of exogenously added ϵ subunit to inhibit the enzyme. In contrast, MTSL labeling of the ϵ-replete F_1 remains inhibited. We hypothesize that a bulky hydrophobic adduct of γT106 blocks proper interactions with the ϵ subunit. The inhibition of ATPase activity by ϵ is abrogated in the γE224C mutant. However, unlike the wild-type enzyme, which has significantly higher activation energy, E_A, for steady state ATPase activity, γE224C mutant F_1 has the same E_A as the ϵ-replete WT enzyme. The results indicate that efficient energy coupling of the γ subunit mutant enzyme is retained. Crystal structure of the E. coli γ-ϵ dimer (Rogers, A. J. W. and Wilce, M. C. J., *Nat. Struct. Biol.* 7, 1051-1054, 2000) suggests that replacement of γThr106 and γGlu224 may disrupt interactions with the C-terminal helix-turn-helix domain of the ϵ subunit. Our data suggest that the ϵ subunit carboxyl terminal domain is responsible for inhibition of catalytic activity also but also plays a role in mediating proper interactions between the γ and *c* subunits and efficient coupling.

2509-Pos Board B495
Unravelling the Symmetry Mismatch of the Two Coupled Rotary Motors of a Single F_OF_1-ATP Synthase by Three Color Fret
Michael Börsch.
F_oF_1-ATP synthase is the enzyme that provides the 'chemical energy currency' adenosine triphosphate, ATP, for living cells. The enzyme consists of the membrane-embedded F_o part and the F_1 part with three catalytic nucleotide binding sites. The formation of ATP is accomplished by a stepwise internal rotation of subunits within both parts. During ATP synthesis, proton translocation through the F_o part drives a 10-step rotary motion of the c_{10} ring with respect to the non-rotating subunits *a* and b_2 [1]. This rotation is transmitted to the γ and ϵ subunits of the F_1 part which perform three 120° steps per full rotation [2]. To localize transient elastic energy storage within the enzyme due to the symmetry mismatch, we monitor subunit rotation by a single-molecule fluorescence resonance energy transfer (FRET) approach using three fluorophores specifically attached to the enzyme. Cy5 is attached to one *c* subunit and EGFP to the *a* subunit of the F_o part [3]. The F_1 part has a fluorophore bound at the ϵ subunit. To reduce photophysical artifacts due to spectral fluctuations of the fluorophores, a duty cycle-optimized alternating three laser scheme (DCO-ALEX) has been developed. Simultaneous observation of the stepsizes for both motors in a single enzyme revealed elastic deformations in the rotor parts of F_o and F_1 during catalysis.
[1] M. G. Düser, N. Zarrabi, D. J. Cipriano, S. Ernst, G. D.Glick, S.D. Dunn, M. Börsch (2009) EMBO Journal 28, 2689-2696.
[2] B. Zimmermann, M. Diez, N. Zarrabi, P. Gräber, M. Börsch (2005) EMBO Journal 24, 2053-2063.
[3] M. G. Düser, Y. Bi, N. Zarrabi, S.D. Dunn, M. Börsch (2008) J. Biol. Chem. 283, 33602-33610.

2510-Pos Board B496
Revisiting Boyer's Hydronium Hypothesis: On the Mechanism of Proton Binding to ATP Synthase Membrane Rotors
Vanessa Leone, Alexander Krah, José D. Faraldo-Gómez.
A recently determined atomic structure of an H^+-transporting ATP synthase membrane rotor has revived the long-standing question of whether protons may be bound to these structures in the form of a hydronium ion. Using both classical and quantum-mechanical simulations, we show that this notion is implausible. *Ab initio* molecular dynamics simulations of the binding site demonstrate that the putative H_3O^+ deprotonates within femtoseconds. The bound proton is thus transferred irreversibly to the carboxylate side chain found in the ion-binding sites of all ATP synthase rotors. This result is consistent with classical simulations of the rotor in a phospholipid membrane, on the 100-nanosecond time-scale. These simulations show that the hydrogen-bond network seen in the crystal structure is incompatible with a bound hydronium. The observed coordination geometry is shown to correspond instead to a protonated carboxylate and a bound water molecule. In conclusion, this study underscores the notion that binding and transient storage of protons in the membrane rotors of ATPases/synthases occur through a common chemical mechanism, namely carboxylate protonation.

2511-Pos Board B497
Side-Chain Carboxylate Needed for Nonstoichiometric Proton Leak through Na/K-ATPase Pumps
Natascia Vedovato, David C. Gadsby.
Cyclic, stoichiometric exchange of 3 Na ions for 2 K ions per ATP hydrolyzed generates outward Na/K-ATPase pump current. With no external Na (Nao) and K (Ko), a nonstoichiometric inwardly-rectifying current flows through Na/K-pumps, proposed carried by protons because it is increased when pH is lowered. Stoichiometric and nonstoichiometric currents are both abolished by the Na/K-pump inhibitor ouabain. In *Xenopus* pumps with ouabain-resistance mutation C113Y, nonstoichiometric current appeared even at pH 7.6 in the absence of Nao and Ko, was augmented ~4 fold at pH 6, was abolished by Ko, and was diminished by high Nao. We suggested that alternate-side exposure of

a protonatable side chain in E2P-like conformations might allow shuttling of protons across the membrane. We therefore evaluated the role of three key carboxylates, E336 (TM4), E788 (TM5), and D935 (TM8), by mutating C113Y *Xenopus* pumps. Outward Na/K-pump current was practically abolished in E336Q(C113Y) or E336C(C113Y) pumps, but nonstoichiometric inward current at zero Ko and Nao (replaced by TMAo) was little altered, and was greatly augmented in high Nao. E788C(C113Y) pumps, on the other hand, like parent C113Y pumps, generated robust stoichiometric outward Na/K transport currents (voltage dependent in Nao, but not in TMAo), and little nonstoichiometric inward current in high Nao; but nonstoichiometric current in TMAo was diminished ~3-fold compared to parent C113Y pumps. In contrast, D935N(C113Y) pumps generated stoichiometric outward Na/K transport currents with altered voltage dependence that was similar in Nao or in TMAo, but inward nonstoichiometric current was nearly absent both in Nao and in TMAo, and was not augmented by lowering pH to 6 either in Nao and in TMAo. The D935 carboxylate thus seems uniquely required for the nonstoichiometric inward flow of protons through the Na/K-pump. [NIH HL36783].

2512-Pos Board B498

Single-Molecule Studies of the Na^+/K^+-ATPase

Promod R. Pratap, Gregor Heiss, Martin Sikor, Don C. Lamb, Max Burnett.

The Na^+/K^+-ATPase, a membrane-associated ion-motive ATPase, uses energy from the hydrolysis of ATP to move Na^+ out of and K^+ into cells. We have labeled ATPase isolated from duck supraorbital salt glands with Cy3-maleimide (Cy3-ATPase). We have previously found that the fluorescence of Cy3-ATPase decreases in the presence of ATP (*Biochim Biophys Acta* 2009; **1794**:1549-1557). We found there that the kinetics of this ATP decrease exhibited negative cooperativity. To determine whether this behavior is due either to interaction between protomers or is an intrinsic property of monomers, we examined the fluorescence of the labeled enzyme solubilized in the nonionic detergent $C_{12}E_8$ in the absence and presence of varying concentrations of ATP using single-molecule total internal reflection spectroscopy (SM-TIRF). We found that: (i) our Cy3-maleimide labeling protocol yielded a significant fraction of singly-labeled protein; and (ii) even with solubilization, a significant fraction of the protein exhibited aggregation. A preliminary analysis of the data from single (non-aggregating) molecules using a hidden Markov model (HMM) suggests a difference in the single-molecule dynamics of the enzyme in the presence and absence of ATP. The implication of these observations will be discussed.

2513-Pos Board B499

A Novel Endogenous Cardiotonic Hormone from Mammalian Muscle and Kidney

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The cardiotonic receptor on the Na,K-ATPase and an endogenous ligand play a physiological role in regulating its enzyme activity in mammalian skeletal muscle (Radzyukevich et al. 2009. *PNAS* 106(8):2565-70). The goal of this study was to develop an approach to identify the endogenous ligand(s). The regulation is preserved *in vitro* and crude muscle extracts retain active factor(s). Therefore, we used muscle as a source of concentrated bound ligand, in combination with a batch affinity purification method which uses the receptor itself to capture its ligands. We applied affinity extraction with a purified Na,K-ATPase to a MeOH extract from skeletal muscle. Ligands were bound to the receptor in Mg^{2+}-containing buffer and released in EDTA. The efficiency of ligand binding was confirmed and optimized using ^{3}H-ouabain. Binding of Mg^{2+} to a single Mg binding site enhances binding at the cardiotonic receptor site by a factor of 215, and thereby favors specific over potentially non-specific ligand binding. The resulting compounds were desalted on hydrophobic SPE cartridge and separated on HPLC with a C18 column. One of the fractions from muscle showed a single peak in MS (m/z 381 M + Na^+). This compound is distinct from ouabain (m/z 606), ouabagenin (m/z 460), marinobufagenin (m/z 423) and other known cardiotonic steroids. We obtained the same factor from pig kidney, plus 3 additional compounds. These results demonstrate that at least one novel cardiotonic hormone is common to different mammalian species and tissues. Given the broad scientific and therapeutic implications of the cardiotonic hormones, our current goal is to accumulate a sufficient amount of this compound for structural identification by cryo NMR. This goal is feasible using a scaled up procedure which yields nanogram amounts (in ouabain equivalent units) per run.

2514-Pos Board B500

Na Pump E960 Site is Critical for the Interaction with Phospholemman

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Phospholemman (PLM), a FXYD family member, critically modulates Na pump (NKA) and thus is important in Na and Ca regulation in cardiac myocytes. PLM inhibits NKA by reducing its apparent Na affinity, effect relieved by PLM phosphorylation. However, the sites responsible for the NKA-PLM interaction are unknown. Based on the recent NKA crystal structure with the associated FXYDs, we constructed single-site CFP-NKAα1 mutants with alanine substitution at F956, E960, L964 and F967 sites. Mutated CFP-NKAα1 and wild-type (WT) PLM-YFP were co-expressed in HEK cells and their interaction was assessed using fluorescence resonance energy transfer (FRET). FRET efficiency was determined as the relative increase in donor fluorescence (ΔF_{CFP}) upon acceptor photobleaching. The NKA-PLM FRET was not altered by the F956A and F967A mutations, was significantly reduced for L964A (ΔF_{CFP} =6 ± 1% *vs.* 17.9 ± 2% for WT-NKA) and was abolished for E960A (ΔF_{CFP} =0.1 ± 1%). Alanine mutation of the PLM site F48, shown by X-ray crystallography to be within interaction distance of NKA E960, eliminated the FRET with WT NKA (ΔF_{CFP}=0.2 ± 4%). Mutation of the same site to cysteine, as in some FXYD proteins, did not affect the NKA-PLM FRET (ΔF_{CFP}=18.5 ± 1.7%). To determine whether PLM affects the function of the E960A NKA mutant, we measured the NKA-mediated Na-extrusion as a function of $[Na]_i$ in a cell line that stably expresses E960A-NKA, with and without WT PLM. The apparent NKA-E960A affinity for $[Na]_i$ was similar in the absence (K_d=9.7 ± 1.0 mM) and in the presence (K_d=8.9 ± 1.7 mM) of WT-PLM and was not significantly affected by forskolin-induced PLM phosphorylation. In contrast, WT-PLM reduced the Na-affinity of WT-NKA (K_d increased from 9.3 ± 1.6 to 11.5 ± 1.9 mM) and PLM phosphorylation lowered the K_d to 9.8 ± 1.8 mM. Thus, our results demonstrate that the E960-F48 interaction is critical for the PLM-NKA association.

2515-Pos Board B501

Uncoupled Inward Currents through Native Na/K Pumps in Guinea Pig Ventricular Myocytes

Camila Zugarramurdi, Juan J. Ferreira, **Pablo Artigas**.

The Na/K pump is a P-type ATPase that maintains essential electrochemical gradients for Na^+ and K^+ across the plasmalemma of animal cells. Within its transmembrane domains the pump presents 3 ion-binding sites, two of which can bind Na^+ or K^+ (shared sites) and another that exclusively binds Na^+ . The mechanisms by which different ions are selected by each site are not fully understood.

Electrophysiological studies from several laboratories investigating the function of Na/K pumps in *Xenopus* oocytes have shown that, without external Na^+ or K^+, the pump passively imports protons and possibly guanidinium$^+$ and its derivatives. It is thought that both protons and guanidinium-derivatives are transported through the Na^+ -exclusive site when the shared sites are empty. Because ion-binding sites of Na/K pumps across the animal kingdom are conserved, it is puzzling that these currents have not been reported in classical preparations where native Na/K pumps have been studied under voltage clamp. Here, we describe these uncoupled inward currents through the native Na/K pumps of Guinea pig ventricular myocytes, demonstrating they are not an artifact of the oocyte system.

Under whole-cell patch-clamp with internal conditions promoting maximal Na/K pump phosphorylation (50 mM Na^+_i, 5 mM MgATP), cardiotonic steroid-sensitive inward currents (I_{unc}) were not observed in 150 mM Na^+_o (at all pH_o) or in 150 NMG^+_o (at pH_o =7.4). In contrast, at negative voltages, large currents I_{unc}=-1,7 ± 0,26 pA/pF (at −180 mV, n=10) were observed in NMG^+_o solutions with pH_o = 6. Also consistent with observations reported in oocytes, inward currents I_{unc} = −1,08 ± 0,14 pA/pF (n=14) were observed in 150 mM guanidinium^+_o (pH_o =7.4). The effects of other Na/K pump ligands on I_{unc} are underway. Financed by TTUHSC SABR and AHA BGIA2140172.

2516-Pos Board B502

The Sodium Pump is Confined in a Phosphoenzyme Form by Lead(II) Ions

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Steady-state fluorescence spectroscopy was used to investigate the effect of Pb^{2+} ions on the Na^+,K^+-ATPase. Experiments were performed by using the electrochromic styryl dye RH421 (1, 2) to characterize in detail the effect of Pb^{2+} ions on the Na-pump and to pinpoint the reaction step(s) of the enzymatic cycle at which the heavy-metal ions provoke their action.

We recently found that Pb^{2+} ions completely inhibit enzyme activity at concentrations above 10 μM (K_I = 0.5 μM (3)). It is now shown that Pb^{2+} ions can bind reversibly to the protein and do not affect the Na^+ and K^+ binding affinities in the E_1 and P-E_2 conformations of the enzyme. This indicate that Pb^{2+} binding to the protein does not block the access pathway to ion binding sites. We also found that lead(II) favors binding of one H^+ to the P-E_2 conformation in the absence of K^+. A model scheme is proposed that accounts for the experimental